Iniparib non-selectively modifies cysteine-containing proteins in tumor cells and is not a *bona fide* PARP inhibitor

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Running title: Iniparib is not a PARP-1 inhibitor

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The abbreviations used are: TMZ, Temozolomide; PARP, poly (ADP-ribose) polymerase; BER, base excision repair; MMR, mismatch repair; NER, nucleotide excision repair; HR, homologous recombination; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; NHEJ, non-homologous end joining.
Statement of translational relevance

PARP inhibitors represent the most clinically advanced examples of agents that specifically target DNA repair for the treatment of cancer. Clinical proof-of-concept for this approach has been reported with the activity of some of these agents in BRCA deficient breast and ovarian cancer. Significant potential also exists for the use of these agents in combination with DNA damaging agents in a variety of tumor types. However, different potential mechanisms of action for inhibition of PARP have been reported amongst these compounds. In particular, iniparib is reported to inhibit PARP by interacting with the zinc finger DNA binding domain of PARP1, rather than competitively interacting with the NAD$^+$ binding site of PARP1/2. Here, we characterize iniparib in enzymatic, cellular, and in vivo assays and demonstrate that this compound does not appear to be a bona fide PARP inhibitor. These results have significant translational relevance with regard to the interpretation of the clinical results with iniparib relative to that observed with other PARP inhibitors.

Abstract

Purpose: PARP inhibitors are being developed as therapeutic agents for cancer. More than six compounds have entered clinical trials. The majority of these compounds are NAD$^+$-competitive inhibitors. One exception is iniparib which has been proposed to be a non-competitive PARP inhibitor. Here we compare the biological activities of two different structural classes of NAD$^+$-competitive compounds with iniparib and its C-nitroso metabolite.
Experimental design: Two chemical series of NAD\textsuperscript{+}-competitive PARP inhibitors, iniparib and its C-nitroso metabolite were analyzed in enzymatic and cellular assays. Viability assays was carried out in MDA-MB-436 (BRCA1 deficient) and DLD1-/- cells (BRCA2 deficient) along with BRCA proficient MDA-MB-231 and DLD1+/+ cells. Capan-1 and B16F10 xenograft models were used to compare iniparib and veliparib in vivo. Mass spectrometry and \textsuperscript{3}H-labeling method were used to monitor the covalent modification of proteins.

Results: All NAD\textsuperscript{+}-competitive inhibitors demonstrate robust activity in a PARP-cellular assay, strongly potentiate the activity of temozolomide, and elicit robust cell killing in BRCA-deficient tumor cells in vitro and in vivo. Cell killing was associated with an induction of DNA damage. In contrast, neither iniparib nor its C-nitroso metabolite inhibited PARP enzymatic or cellular activity, potentiated temozolomide, or showed activity in a BRCA-deficient setting. We find that the nitroso metabolite of iniparib forms adducts with many cysteine-containing proteins. Furthermore, both iniparib and its nitroso metabolite form protein adducts non-specifically in tumor cells.

Conclusions: Iniparib non-selectively modifies cysteine-containing proteins in tumor cells and the primary mechanism of action for iniparib is likely not via inhibition of PARP activity.
Introduction

Poly (ADP-ribose) polymerase-1 (PARP-1) is the founding member of a family of proteins that share a catalytic PARP homology domain and are characterized by their ability to poly (ADP-ribosyl)ate protein substrates (1-3). Of the eighteen PARP family members identified to date (1, 2), PARP-1 and PARP-2 are unique in exhibiting stimulation of catalytic activity in response to DNA damage (4). Activation of PARP-1 and PARP-2 is an immediate eukaryotic cellular response to DNA damage induced by a variety of stimuli including ionizing radiation, alkylating agents and oxidants (2). Activated PARP-1/2 bind to DNA strand breaks, and covalently attach poly(ADP-ribose) to nuclear proteins including PARP-1 itself, histones, and transcription factors (1). The enzymatic product of PARP, poly (ADP-ribose) (PAR), is generated from its substrate β-nicotinamide adenine dinucleotide (NAD⁺) and consists of linear and branched ADP-ribose units of variable size (5).

A number of PARP inhibitors have entered clinical trials over the last several years to investigate the effectiveness of these agents in various oncology settings (6). Most of these small molecule inhibitors are based on a nicotinamide-like pharmacophore, owing to their mechanism as competitive binders to the NAD⁺ pocket within PARP-1/2 (6). The more advanced of these compounds display single-digit nanomolar IC₅₀s for both PARP-1 and PARP-2 (6). The preclinical activity of several of these compounds has been described in detail (7-10). As expected, these agents interfere with the base excision DNA repair (BER) process, leading to enhanced tumor cell killing when administered in combination with various types of DNA damaging agents (7-9, 11). In particular, PARP
Inhibitors have been shown to significantly enhance the activity of alkylating agents such as temozolomide in multiple tumor types, due to the importance of BER in repairing adducts formed by these agents (8, 11). Preliminary evidence for clinical activity of this combination has also been reported (12).

In addition, PARP inhibitors have been shown to have single agent activity in tumors that are defective in double-strand break (DSB) repair. The basis for this “synthetic lethality” stems from the fact that inhibition of BER leads to an accumulation of single strand DNA damage that, in turn, results in an elevated level of DSB damage. In the absence of functional DSB repair capacity, the accumulation of DSB damage results in cell death. The most notable example of this phenomenon is in tumor cell lacking BRCA1 or BRCA2. Preclinical studies with multiple NAD⁺-competitive PARP inhibitors demonstrated robust tumor cell killing both in vitro and in vivo in tumors lacking functional BRCA activity (13, 14). Furthermore, clinical proof-of-concept for this synthetic lethality has been established in Phase II studies with olaparib where overall response rates of 33% and 41% were reported in BRCA-deficient ovarian and breast cancer, respectively (15, 16).

In contrast, one purported inhibitor is distinct from the other described compounds in both structural class and proposed mechanism of action. The benzamide, iniparib, is reported to be a pro-drug whose C-nitroso metabolite, 4-iodo-3-nitrosobenzamide, selectively kills tumor cells by oxidizing the zinc finger of PARP-1 resulting in ejection of zinc and inhibition of PARP activity (17). Iniparib or its metabolites have been reported to show single agent activity in certain tumor cell lines, although preclinical
activity in \textit{BRCA}-deficient lines has not been reported (17, 18). Combination activity has also been reported \textit{in vivo}, most notably with platinums and nucleoside analogs in models of triple-negative breast cancer (19, 20). A Phase II study examining the combination of iniparib with gemcitabine and carboplatin in the triple-negative setting produced a significant 3.5 month improvement in overall survival relative to the cytotoxic regimen alone (21). However, activity in the first line setting was not recapitulated in a recently completed Phase III study; however, it is noteworthy that this agent was well-tolerated and that the compound did appear to show some activity in the second and third line setting (22).

Here, we directly compare the biological activities of two different structural classes of NAD$^+$-competitive PARP inhibitors with iniparib and its c-nitroso metabolite. All of the potent competitive inhibitors demonstrated activity in a PARP-driven cellular assay, potentiated the activity of temozolomide, and elicited cell killing in \textit{BRCA}-deficient tumor cells both \textit{in vitro} and \textit{in vivo}. Cell killing was associated with induction of DNA damage. In contrast, neither iniparib nor its C-nitroso metabolite inhibited PARP enzymatic or cellular activity, potentiated temozolomide, or showed activity in a \textit{BRCA}-deficient setting. We find that the nitroso metabolite of iniparib is highly reactive, forming covalent interactions with many cysteine containing proteins. Furthermore, both iniparib and its nitroso metabolite form protein adducts non-specifically in tumor cells. These results suggest that the primary mechanism of action for iniparib is likely not via inhibition of PARP activity, which will have important implications for the clinical development of this compound.
Materials and Methods

Chemicals: Veliparib, cmpd A-C, iniparib, and iniparib-met were synthesized at Abbott Laboratories. TMZ was purchased from Dik Drug Co. (Burr Ridge, IL). All other chemicals were from Sigma (St. Louis, MO).

Measurement of protein concentration: Protein concentrations were determined using the BCA method (Pierce, Rockford, IL).

Cell lines: DLD1 BRCA2+/+ and DLD1 BRCA2 Δex11/Δex11 (designated as DLD+/+ and DLD-/- cells, respectively) human isogenic cell lines were obtained from Horizon Discovery Ltd, Cambridge, UK and engineered using homologous recombination mediating rAAV gene targeting vectors. MDA-MB-231, Capan-1, B16F10 and MDA-MB-436 cells were obtained from the American Type Culture Collection (Rockville, MD).

PARP enzymatic assay: PARP-1 enzymatic assays were carried out for veliparib, cmpd-A, cmpd-B, cmpd-C, iniparib and iniparib-met. PARP-2 assays were carried out for veliparib, cmpd-A, cmpd-B. PARP enzymatic assays were conducted in buffer containing 50 mM Tris pH 8.0, 1mM DTT, 1.5 μM [³H]-NAD+ (1.6 μCi/mmol), 200nM biotinylated histone H1, 200 nM DNA oligo (CACACAGTTGTATCATCTCAGACCTATGCAGAGGAATGCA ACACCTTGTG), and 1nM PARP-1 or 4nM PARP-2 enzyme. Compounds were assayed...
as 11 point, 3-fold dilution series from 10 μM to 170 pM in 2% DMSO. Reactions were carried out in 100 μl volumes in white 96 well plates (Perkin Elmer, Boston MA). These reactions are terminated after 1hr by the addition of 150 μl of 1.5 mM benzamide (~1000-fold over its IC50). 170 μl of the stopped reaction mixtures are transferred to streptavidin Flash Plates, incubated for 16 hr, and counted using a TopCount microplate scintillation counter.

**PARP cellular assay:** C4-I cells (40,000 cells/well) were treated with veliparib, cmpd-A, cmpd-B, cmpd-C and iniparib at 0, 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 μM for 30 min in 96 well plate. PARP was then activated by damaging DNA with 1 mM H2O2 for 10 minutes. The cells were then washed with ice-cold PBS once and fixed with pre-chilled MeOH/Acetone (V/V: 7:3) at -20oC for 10 minutes. After air-drying, the plates were rehydrated with PBS and blocked with 5% non-fat dry milk in PBS-Tween (0.05%) (blocking solution) for 30 minutes at room temperature. The cells were incubated with anti-PAR antibody 10H (1:50) in blocking solution at room temperature for 60 minutes followed by washing with PBS-Tween-20 five times, and incubation with goat anti-mouse FITC-coupled antibody (1:50) and 1 μg/mL DAPI in blocking solution at room temperature for 30 minutes. After washing with PBS-Tween 20 five times, the analysis was performed using an fmax Fluorescence Microplate Reader (Molecular Devices, Sunnyvalle, CA), set at the excitation wavelength of 490 nm and emission wavelength of 528 nm (FITC) or the excitation wavelength of 355 nm and emission wavelength of 460 nm (DAPI). The PARP activity (FITC signal) was normalized with cell numbers (DAPI).
Cell Titer Glow Proliferation Assay: For nine day cell proliferation assay, MDA-MB-436 and MDA-MB-231 cells were plated at 2000 and 500 cells/well respectively in a 96-well plate and treated with veliparib, cmpd-A, cmpd-C, iniparib or iniparib-met at 0, 0.0001, 0.01, 0.1, 1 or 10 µM for nine days. For five day cell proliferation assay, MDA-MB-231 and MDA-MB-436 cells were plated at 1000 and 4000 cells/well respectively in a 96-well plate and treated with iniparib or iniparib-met at 0, 0.1, 0.3, 1, 3 or 10 µM in the presence of 0, 1.8, 3.75, or 7.5 µM BSO for 5 days. DLD1+/+ and DLD1-/- cells were plated at 1000 cells/well in a 96-well plate and treated with TMZ at 0, 0.003, 0.01, 0.03, 0.1, 0.3 or 1 mM in the presence of 0, 0.005, 0.05, 0.5, or 5 µM veliparib, or iniparib for five days. After treatment, cell titer glow was carried out according to manufacturer’s instructions (Promega. Inc., Camarillo, CA).

γH2AX Assay: DLD1+/+ and DLD1-/- cells (0.3 million/well) were treated with DMSO, 0.1 mM TMZ, 5 µM veliparib, or 0.1mM TMZ and 5 uM veliparib for 30 min. Cells treated with DMSO or TMZ alone were pre-treated with DMSO for 30 min. Cells treated with veliparib alone or TMZ/veliparib combination were pre-treated with 5 µM veliparib for 30 min. The cells were then washed and incubated in complete medium containing either DMSO (for DMSO or TMZ alone) or 5 µM veliparib (for veliparib alone or TMZ/veliparib combination) for one hour or 6 hours. Cells were harvested and fixed with 250 µl of BD Cytofix/Cytoperm solution (BD bioscience, San Jose, CA) at room temperature for 20 min followed by washing three times with 1XBD Perm/Wash Solution (BD bioscience, San Jose, CA). After blocking with 3% BSA for 30 min, the cells were incubated with the following antibodies in Perm/Wash Solution with washes in
between: mouse anti-γH2AX antibody (1:250) (Upstate Biotechnologies, NY) and Alexa 488 goat anti-mouse IgG(H+L)F(ab’)2 fragment conjugate (1:200) (Invitrogen, Carlsbad, CA). The DNA was immunostained with 50 μg/mL propidium iodide (PI) in staining buffer [PBS without Mg^{2+} or Ca^{2+}, 1% heat-inactivated FCS, 0.09% (w/v) sodium azide, PH to 7.4-7.6] for 10 min in the dark. Immuno-flowcytometry was performed to quantify the γH2AX signal. Each data point is the average of two values.

**Colony formation assay:** DLD1 BRCA2^{+/+}, DLD1 BRCA2^{Δex11/Δex11} cells (200 cells/well) were seeded into 6 well plates. The cells were treated with veliparib and iniparib at 0, 0.001, 0.01, 0.1, 1 and 10 μM for 11 days. The colonies were stained with Giemsa stain according to manufacturer’s instructions (Sigma, MO). The total area of colonies was scored by counting the colonies using the GelCount system (Oxford, UK).

**In vitro PARP auto-modification assay:** The PARP-1 auto-modification assay was adapted from method reported previously using the same double stranded DNA sequence obtained from Integrated DNA technologies (Skokie, IL) (23). Briefly, PARP-1 was incubated with DMSO, 110 μM iniparib, 110 μM iniparib-met, or 110 μM cmpd-B for 10 minutes followed by addition of double stranded DNA for 20 minutes. NAD^{+} was subsequently added to the reaction mixture for an additional 15 minutes. All steps were carried out at RT and the reaction was halted by addition of 90 mM EDTA and Laemmlli reducing sample buffer. Final concentrations of PARP-1, compound, DNA, and NAD^{+} were: 740 nM, 110 μM, 7 μM, 5.5 mM, respectively. 10 μl of the reaction mixture was loaded onto an SDS-PAGE gel. Results were visualized by Coomassie staining.
Recombinant protein expression: The cloning, expression and purification of PARP-1 zinc finger domain-1 was carried out as described (24). The DNA sequence encoding amino acid 223-324 of Human La antigen was cloned into pAG5 vector with a C-terminal his-tag and expressed in BL21(DE3)/pLysS (25). The protein was purified with a Ni-NTA column.

Detection of covalent compound addition by mass spectrometry: To monitor covalent additions of compound to protein, 1 µM protein was incubated with 200 µM compound for two hours at RT in the presence of 50 mM HEPES and 100 mM KCl. Intact protein molecular weights were measured by mass spectrometry. LC-ESI-MS was performed using an Agilent 6510 QTOF MS coupled to an Agilent 1100 capillary HPLC loading pump and a 1200 nano-HPLC gradient pump with a Chip Cube (40 nL enrichment column, 43 mm x 75 µm analytical column, Zorbax 300SB-C8). The samples were loaded with 0.1% formic acid in 4.5% acetonitrile with a flow rate of 4 µL/min. The gradient nano-pump used A composition (0.1% formic acid in 97% water, and 3% acetonitrile) and B composition (0.1% formic acid in 97% acetonitrile) with a flow rate of 400 nL/min. The nano-pump gradient was set to 3-75% B until 2 minutes, hold at 75% B until 5 minutes, 75-3% B until 5.5 minutes, and hold at 3% B until 8 minutes. Data was acquired with MassHunter B.02.00 and deconvoluted with MassHunter B.04.00.

Capan-1 xenograft tumor study: C.B.-17 SCID female mice were obtained from Charles River (Wilmington, MA). Mouse body weight at initiation of therapy was ~21 g.
Capan-1 brei was prepared by homogenizing 30 g of tumor tissue with 30 mL media. Matrigel was added to the mixture (1:1) to complete the brei. An inoculation volume of 0.2 mL was injected into the right hind flank of the C.B.-17 SCID female mice on day 0. Single agent therapy was administered PO, bid x 14 days for veliparib and IP, bid x 14 days for iniparib starting from day 25. Tumor volume was calculated twice weekly. Measurements of the length (L) and width (W) of the tumor were taken via electronic caliper and the volume was calculated according to the following equation: $V = \frac{L \times W^2}{2}$ using Study Director version 1.7.39 (Studylog Systems, Inc, South San Francisco). The percent tumor growth inhibition (% TGI) was calculated using the formula $100 - \% \frac{T}{C}$ (drug treated/ vehicle-treated tumor volume x 100). Data were analyzed using the Student's t-test for differences in T/C values (StatView, SAS Institute, Cary, NC).

**B16F10 xenograft tumor study:** C57BL/6 female mice were obtained from Charles River (Wilmington, MA). Mouse body weight at initiation of therapy was ~22 g. A total of 6 x 10^4 viable B16F10 cells were inoculated subcutaneously into the right flank of female C57BL/6 mice on day zero. The injection volume was 0.1 mL and was composed of a 1:1 mixture of S-MEM and matrigel. Veliparib was administered PO bid x 5 days and iniparib was administered IP bid x 5 days starting from day 7. TMZ was administered PO qd x 5 days. Tumor volume was calculated three times weekly. Tumor volume calculation, % TGI calculation and data analysis are the same as Capan-1 xenograft tumor study.
Synthesis of $^3$H-iniparib and $^3$H-iniparib-met: Iniparib was radiolabeled by catalytic tritium/hydrogen isotope exchange using tritium gas and Crabtree’s catalyst after the method of Hesk et al (26).

Results

Compound activity in PARP enzymatic and cellular assays

The structures of the compounds used in this study are shown in Figure 1A. Veliparib, cmpd-A, and cmpd-B are all benzimidazole carboxamide (benzimidazole) based compounds and cmpd-C is a tetrahydropyridopyridazinone (pyridazinone). Iniparib and its C-nitroso metabolite (iniparib-met) are of the benzamide class. To evaluate the ability of each of these compounds to directly inhibit PARP enzymatic activity, PARP-1 and PARP-2 enzymatic assays were carried out using a DNA-dependent radiometric activity assay. The nature of this assay allows for detection of PARP inhibition by compounds that are NAD$^+$ competitive as well as compounds such as iniparib that are suggested to interact with the PARP-1 zinc finger. As shown in Table 1, the benzimidazole and pyridazinone inhibitors exhibit single digit nM $IC_{50}$’s for both PARP-1 and PARP-2, whereas neither iniparib nor iniparib-met showed any significant activity against PARP-1.

To examine PARP inhibition in a cellular context, we assayed the ability of compounds to inhibit PAR formation following hydrogen peroxide stimulation in C4-I cells. Hydrogen peroxide treatment activates PARP-1 resulting in an elevated PAR signal to background that enables robust detection of PARP-1 inhibition in cells (27). Consistent with their potent enzymatic activities, veliparib and cmpds A-C demonstrated robust,
single-digit nM EC50s in the cellular assay. In contrast, neither iniparib nor iniparib-met were able to effectively inhibit PARP-1 enzymatic activity and iniparib was also inactive in the PARP cellular assay (Table 1).

One of the properties of PARP-1 is its ability to poly-ADP-ribosylate itself (23, 24). As another measure of the ability of compounds to inhibit PARP activity, we examined auto-ribosylation of purified PARP-1 in the presence of double-stranded DNA. In the presence of NAD\(^+\), the potent NAD\(^+\)-competitive cmpd-B completely inhibited auto-ribosylation (Fig. 1B, visualized as a single band). In contrast, a smear was observed in samples treated with iniparib, iniparib-met, or the DMSO control, indicating that iniparib and iniparib-met were unable to inhibit PARP-1 auto-ribosylation to any measurable extent (Fig. 1B).

**Single agent activity in BRCA-deficient tumor cells**

Inhibition of PARP activity, by any mechanism, should result in compromised BER activity that, in turn, leads to synthetic lethality in DSB repair deficient cells (13, 14). To examine this, we utilized two breast cancer cell lines, MDA-MB-436 and MDA-MB-231 with different DSB repair capacity. MDA-MB-436 cells have a mutation in the splice donor site of exon 20 of BRCA1, resulting in defective transcripts (28), while MDA-MB-231 cells have wild type BRCA function (29). In nine day proliferation assays, veliparib, cmpd-A and cmpd-B inhibited MDA-MB-436 cells with EC50s of 96, 105 and 550 nM, respectively (Fig. 2A), whereas iniparib and iniparib-met showed no significant effects on proliferation at concentrations as high as 10 \(\mu\)M (Fig. 2A). None of the compounds
showed significant anti-proliferative activity in the BRCA wild-type MDA-MB-231 cell line at concentrations as high as 10 µM (Fig. 2B).

Since glutathione transferase activity can potentially deactivate the C-nitroso metabolite of iniparib, it has been reported that the cellular cytotoxicity of the iniparib-met can be enhanced by co-treatment with the GSH inhibitor buthionine sulfoxamid (BSO) (17).

To determine if the cellular activity of iniparib and iniparib-met was attenuated by glutathione transferase activity, we examined the anti-proliferative activity of these compounds in the presence or absence of BSO in MDA-MB-436 and MDA-MB-231 cells. In MDA-MB-436 cells the addition of BSO to iniparib-met did not lead to any appreciable decrease in cell proliferation (Fig. 2D); however, a modest effect was observed when BSO was added to these cells treated with 10 µM iniparib (Fig. 2C). The addition of BSO to iniparib and iniparib-met produced a more significant effect on proliferation in MDA-MB-231 cells; however, BSO alone also appeared to produce some cytotoxicity in these cells (Fig. 2E, 2F).

In order to more rigorously evaluate the activity of these compounds in a DSB repair deficient setting, we utilized an isogenic pair of tumor cell lines in which one line (DLD1 \( BRCA2^{+/+} \)) is functional for BRCA2 activity while the other (DLD1 \( BRCA2^{Δex11/Δex11} \)) is defective in BRCA2 activity (hereafter referred to as DLD+/+ and DLD-/- cells, respectively) (30). In colony formation assays, veliparib was found to inhibit proliferation of the BRCA2 defective DLD1-/- cell line with an EC\(_{50}\) of 0.04 µM while no effect was observed in the BRCA2 wild-type DLD1+/+ cell line (Fig. 2G). In contrast, iniparib had no effect on either cell line at concentrations as high as 10 µM (Fig. 2H).
Combination activity with temozolomide (TMZ)

As PARP-1/2 play critical roles in BER, inhibition of PARP-1/2 should enhance the efficacy of cancer therapeutics that cause DNA damage requiring BER-mediated repair. TMZ is a mono-functional DNA alkylating agent that induces DNA damage requiring BER-mediated repair and is used for the treatment of melanoma and glioma. Numerous pre-clinical studies have demonstrated the ability of PARP inhibitors to potentiate the activity of TMZ in both in vitro and in vivo model settings (7, 8, 11). This activity is particularly pronounced when used in tumors defective in DSB repair (8, 31).

We therefore examined the ability of these compounds to enhance the activity of TMZ in BRCA-defective MDA-MB-436 cells and a DLD1+/+ and DLD1-/- isogenic pair as a means of exploring PARP inhibitor-mediated combination activity.

As shown in Fig 3A-E, veliparib, cmpd-A and cmpd-C were able to potentiate TMZ (5.2, 4.2, and 2.4-fold respectively) at concentrations as low as 50 nM in MDA-MB-436 cells in 5 day proliferation assays, while iniparib and iniparib-met did not show any enhancement of TMZ cytotoxicity when used at concentrations as high as 5 µM.

In the DLD1 isogenic pair, veliparib was able to potentiate the activity of TMZ in both BRCA2 wild-type and BRCA2 mutant cells with the effect being more pronounced in the mutant cells, requiring a lower concentration of veliparib (50 nM vs 5 µM) (Fig.3F-G).

In contrast, no enhancement of TMZ activity was observed in either cell line with concentrations of iniparib as high as 5 µM (Fig.3H-I). We have also examined both TMZ/veliparib and TMZ/iniparib in HR competent cell lines including MDA-MB-231, HCT116. In all cases, veliparib, but not iniparib, potentiated the toxicity of TMZ (data not shown).
Induction of γH2AX is a widely used measure of DSB formation and we have demonstrated previously that cell cytotoxicity is correlated with the induction of γH2AX signal in HCT116 cells treated with veliparib plus TMZ (11, 32). We used this same approach to monitor the induction of DNA damage in the DLD1 BRCA2 isogenic pair. As expected, DLD1-/- cells have a higher background level of γH2AX signal than DLD1+/+ cells owing to the deficiency in DSB repair (Fig.4A). When examined 1hr after treatment, TMZ or veliparib alone did not significantly induce γH2AX signal in either DLD1+/+ or DLD1-/- cells, while TMZ/veliparib induced γH2AX signal in both lines (Fig.4A). DLD1+/+ cells were able to repair DSB’s after a six hour recovery as indicated by a return of γH2AX signal to baseline levels (Fig.4B). In contrast, γH2AX levels remained elevated in DLD1-/- cells up to six hour after treatment (Fig. 4B). This result indicates that the enhanced cell killing produced by the addition of veliparib to TMZ is associated with increased DNA damage due to attenuated BER capacity. This effect is significantly more pronounced in cells that are also compromised for DSB repair activity. In this experiment, treatment with veliparib alone did not induce a significant γH2AX signal in DLD1-/- cells owing to the fact that these cells were treated for only 1 hr. Treatment of DLD1-/- cells with 10 µM veliparib for 72 hr did result in a significant increase in γH2AX signal (22% vs 5% for DMSO control). In contrast, no effect was observed with iniparib treatment under similar conditions (5% vs 5% for DMSO control) (data not shown).

Activity in xenograft tumor models
To extend our *in vitro* analyses of these compounds to the *in vivo* setting, we examined the activity of several compounds in two xenograft models that have been extensively characterized for their response to PARP inhibitors. Capan-1 is a BRCA2-deficient human pancreatic cancer cell line in which NAD⁺ competitive PARP inhibitors, olaparib and AG014699, were reported to show significant single agent activity in both tissue culture and tumor xenograft models (29, 33). When given orally at doses of 200 mg/Kg/day (100 mkd, twice daily) for 14 days, veliparib produced a significant 53% tumor growth inhibition (TGI) at day 59 compared to the vehicle group in the capan-1 model (Fig. 5A). Various dose and schedules have been reported for the use of iniparib in preclinical models, most typically utilizing a dose of 50 mg/Kg/day given IP on an intermittent dosing schedule (19, 20). Consistent with previous reports of rapid and extensive metabolism of iniparib *in vivo*, we were unable to detect any presence of the parent molecule in mouse pharmacokinetic studies (data not shown). In tolerability studies in C.B.-17 SCID mice, doses above 100 mg/Kg/day, once daily, IP, were not tolerated. In the capan-1 model, no significant tumor growth inhibition was observed when iniparib was given at the maximum tolerated dose of 100 mg/Kg/day (IP, 50 mkd, twice daily) for 14 days compared to the vehicle control (16% TGI at day 59; Fig. 5A).

We and others have demonstrated previously PARP inhibitors can effectively potentiate the activity of TMZ in various xenograft models (7, 8, 31, 34). The doses of veliparib that are sufficient to produce anti-tumor activity have also been shown to be pharmacodynamically active, leading to significant inhibition of PAR levels *in vivo* (8, 31, 35, 36). Consistent with our previous reports, TMZ monotherapy at 50 mg/Kg/day (IP, once daily for five days) produced significant tumor growth inhibition (45% TGI at...
day 14) when compared to the vehicle group (Fig. 5B). Veliparib, given orally at 25 mg/Kg/day (12.5 mkd, twice daily) for 5 days, in combination with TMZ, was able to significantly enhance tumor growth inhibition (58% TGI at day 14) relative to TMZ monotherapy (Fig.5B). Similar effects were observed for cmpd-A (61% TGI at day 15 at 30 mg/Kg/day), cmpd-B (49% TGI at day 14 at 10 mg/Kg/day) and cmpd-C (57% TGI at day 14 at 60 mg/Kg/day) (data not shown). In contrast, iniparib, given IP at doses 100, 50, or 25 mg/Kg/day (50, 25, or 12.5 mkd, twice daily) for 5 days in combination with TMZ did not produce significant improvement in anti-tumor activity relative to TMZ monotherapy (9, 9, and 0% TGI respectively, at day 14) (Fig.5B).

The C-nitroso metabolite of iniparib reacts non-specifically with proteins containing cysteine residues

The data described above indicate that neither iniparib nor its C-nitroso metabolite demonstrates the biological activity one would expect from inhibition of PARP function. The structure of iniparib-met suggests that this molecule is highly reactive and likely to interact non-specifically with many proteins containing cysteine residues. To test this, we incubated iniparib-met with several proteins containing cysteine residues including La Antigen (25), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), PARP-1 zinc finger-1, or ubiquitin, a protein that does not contain cysteine. We used DMSO as a negative control and tested the ability of cmpd-B and iniparib-met to react with these proteins. As shown in Fig. 6, incubation with cmpd-B resulted in no mass shifts as compared to the control, indicating that the structure of each protein was not altered by cmpd-B. In contrast, a series of new masses were observed for La, GAPDH, and PARP-
1 zinc finger-1 when incubated with iniparib-met (Fig. 6). The mass of ubiquitin, a protein lacking cysteine residues, was not affected by treatment with cmpd-C or iniparib-met (Fig. 6). These data indicate that iniparib-met is capable of interacting with the zinc finger domain of PARP-1 as reported (17). However, this appears to be a non-specific interaction as iniparib-met is equally capable of interacting with many other cysteine containing proteins beyond PARP-1.

Iniparib and iniparib-met form non-specific protein adducts in tumor cells

In order to confirm that iniparib and iniparib-met form protein adducts non-specifically in cells, we incubated \(^{3}\)H-labeled iniparib and iniparib-met with MDA-MB-436 and MDA-MB-231 cells in the presence or absence of BSO. The cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes. \(^{3}\)H-labeled proteins were visualized by fluorography. As shown in Fig. 7, both iniparib and iniparib-met were able to form multiple protein adducts in cells. BSO did not enhance significantly the protein adduct formation under the current experimental condition for both iniparib and iniparib-met. Western blot analysis indicated that PARP-1 was present in the cell lysate and was not a major protein to forms adducts with either iniparib or inaparib-met (Fig.7). We also observed that more protein adducts were formed for iniparib than iniparib-met suggesting that iniparib-met may not be the sole mechanistic mediator for iniparib activity in cells (Fig. 7). Together our data suggest that iniparib and iniparib-met react non-specifically with many cysteine-containing proteins. Although we can not rule out that iniparib or iniparib-met interacts with PARP-1 in tumor cells, we were unable to detect a significant interaction in MDA-MB-436 or MDA-MB-231 tumor cells.
Discussion

Many established cancer therapies involve DNA-damaging chemotherapy or radiotherapy. PARP-1 and PARP-2 are nuclear enzymes that are activated by DNA-damage and play a critical role in base excision repair (BER) that occurs in response to damage induced by many such therapeutic modalities. As such, inhibition of PARP represents an attractive approach for the treatment of cancer for use in combination with DNA damaging agents and as a monotherapy in tumor settings deficient in complimentary DNA repair process (7, 8, 13, 14).

As an attractive therapeutic target, multiple compounds are being developed in order to modulate PARP activity. The majority of such compounds are expected to act by an NAD⁺-competitive mechanism targeting both PARP-1 and PARP-2. Iniparib is structurally and mechanistically distinct. Its active metabolites are purported to covalently interact with the zinc finger of PARP-1 disrupting its interaction with DNA and thereby specifically targeting PARP-1 (17). However, regardless of the mechanism of action, inhibition of PARP-1 function should result in compromised BER activity with the accompanying biological responses associated with disruption of this DNA repair process.

At least six PARP compounds are in various stages of clinical development for oncology with several other compounds in pre-clinical development (6). Promising preliminary clinical data has been reported in combination with TMZ, topoisomerase inhibitors, and
cytoxan in Phase I studies (12, 37, 38). Evidence for single agent activity has also been reported in BRCA deficient tumors in Phase I/II studies with both olaparib and MK4827 (15, 16, 39, 40). A Phase II study with iniparib in triple-negative breast cancer indicated very promising activity in combination with gemcitabine and carboplatin (21). However, activity in the first line setting could not be confirmed in a subsequent Phase III study, although the compound was well tolerated and did appear to show some activity in the second and third line setting (22).

In order to investigate potential differences in fundamental mechanisms and hence potential differences in clinical utility for the different classes of PARP compounds, we herein characterize and compare their respective activities in multiple established PARP-specific assays. Specifically, we utilized a variety of enzymatic, cellular and in vivo assays to compare the activity of two distinct structural classes of NAD$^+$-competitive PARP inhibitors with the benzamide, iniparib, and its C-nitroso metabolite, 4-iodo-3-nitrosobenzamide. The NAD$^+$-competitive benzamidazole and pyridazinone-based compounds displayed the expected biological activity associated with PARP inhibition including potent activity in established enzymatic and cellular PARP assays. In addition, these compounds induced synthetic lethality in tumor cells with both endogenous and exogenous BRCA mutations. On the contrary, neither iniparib nor iniparib-met showed any of the expected biological activity associated with inhibition of PARP.

Inhibition of PARP activity is known to enhance the activity of various classes of DNA damaging agents (7, 8). Potentiation of alkylating agents such as TMZ is perhaps the best characterized example of this effect with robust pre-clinical activity reported for
numerous PARP inhibitors (8, 31) and preliminary evidence of clinical activity also reported (12, 41). Consistent with our previously published work in HCT-116 cells, we demonstrated that the addition of veliparib significantly enhanced the activity of TMZ in the DLD1 BRCA isogenic pair and that these anti-proliferative effects were associated with sustained DNA damage in the presence of veliparib (Fig.3 and Fig.4). This effect was more pronounced in the BRCA deficient tumor cell line compared to the BRCA wild-type line (Fig.3). Consistent with this finding, recently reported Phase I/II clinical data suggest that the combination of veliparib plus TMZ is also active in BRCA-deficient breast cancer (41). In contrast, iniparib was unable to significantly potentiate the activity of TMZ in either cell line when used at concentrations as high as 5 µM (Fig. 3).

Moreover, no evidence of enhanced DNA damage, as indicated by increased γH2AX signal, was observed upon treatment of cells with 5 µM iniparib in combination with TMZ or 10 µM as single agent in MDA-MB-346 cells (data not shown). Together, these data suggest that iniparib is mechanistically distinct from NAD⁺-competitive PARP inhibitors with respect to utility in combination with DNA damaging agents or in the context of BRCA deficiency.

To extend the analysis to an in-vivo setting we investigated the activity of these compounds in capan-1 and B16F10 xenograft models in which PARP inhibitor responses have been well-characterized. Capan-1 is a human pancreatic cancer cell line with BRCA2 deficiency and the NAD⁺ competitive PARP inhibitors olaparib and AG014699 have both been reported previously to show significant single agent activity in this cell line (29, 33). Consistent with reported sensitivity to PARP inhibition, veliparib demonstrated significant efficacy in the capan-1 model (Fig. 5). B16F10 murine
melanoma represents a clinically relevant cancer that is relatively resistant to most chemotherapeutics including TMZ. Consistent with our previous data, veliparib and several other NAD⁺ competitive compounds significantly potentiated TMZ efficacy in the B16F10 model (8). In contrast, iniparib did not show significant activity in either model (Fig. 5).

Taken together our data suggest that iniparib is not a PARP inhibitor and that clinical activity of this compound is mechanistically distinct from that of NAD⁺-competitive class of molecules. Indeed, iniparib has been suggested to interact with targets beyond PARP that may account for the reported activity of this compound (42-45). The reactive nature of the iniparib-met molecule suggests that non-specific modification of cysteine containing proteins is likely to occur with this compound. Accordingly, assessment of biochemical reactivity by mass spectrometry detected covalent compound addition not only to the zinc finger domain of PARP-1 but also to several other cysteine containing proteins as well (Fig. 6). Moreover, this general reactivity also pertains to the cellular context where iniparib and iniparib-met were observed to form multiple protein adducts. Importantly, adducts of PARP-1 were not prominent in this study despite the purported mechanism of these compounds and the cellular abundance of PARP-1 protein (Fig. 7).

If iniparib does not functionally inhibit PARP activity in tumor cells, what is the explanation for the reported preclinical and clinical activity of this molecule? We propose that the reported activity of iniparib may result from non-selective modification of numerous proteins. The formation of such non-specific adducts could alter the stability, activity, and/or localization of the respective proteins resulting in a cellular
insult capable of inducing any of various cellular responses including but not limited to apoptosis, stress, cell-cycle perturbation, or DNA-damage. It is plausible that such responses might sensitize the effected cells to cytotoxic therapy such as that observed with iniparib clinically. This non-specific mechanism of action is supported by the ability of iniparib and iniparib-met to form protein adducts with several cysteine-containing proteins, both purified and in a cellular context. This type of mechanism is somewhat analogous to the S-nitrosylation of proteins by nitric oxide. In this signal transduction paradigm, specificity does not result from specific interactions between nitric oxide and target proteins, but rather from localized production of nitric oxide by nitric oxide synthase followed by non-specific nitrosylation of reactive cysteines in the vicinity (46). Indeed, numerous proteins with reactive cysteines have been demonstrated to be S-nitrosylated (46). Perhaps not coincidentally, two proteins that are modified by iniparib-met, PARP-1 and GAPDH, have been shown to be targets of S-nitrosylation (47).

These data have important implications for how these compounds are developed clinically. Although there are no current active clinical trials with iniparib in a specifically selected BRCA population, self-selection for BRCA carriers may be expected to occur in iniparib trials due to the widely understood activity of PARP inhibition in the BRCA mutant setting. Since iniparib appears to function primarily through a mechanism unrelated to PARP inhibition, trials including BRCA deficient patients or combinations designed to exploit inhibition of BER activity may not represent the most effective clinical application. In addition, our data raises concerns about the utilization of exclusion criteria based on the classification of iniparib as a PARP inhibitor.
that may prevent patients who have been treated previously with iniparib to receive subsequent treatment with *bona fide* PARP inhibitors.

In summary, our results clearly show that iniparib acts through a distinct mechanism from classical NAD$^+$-competitive PARP inhibitors such as veliparib. As such, it is likely that clinical utility including potential therapeutic combinations, responsive patient populations, and appropriate clinical biomarkers will not be the same between these distinct drug classes. Therefore, care should be taken not to translate clinical findings across studies using NAD$^+$-competitive PARP inhibitors with those using iniparib.

**Disclosure of Potential Conflicts of Interest**

All authors are employees of Abbott Laboratories. No potential conflicts of interest are disclosed.

**Acknowledgement**

We would like to thank Viraj Gandhi for making iniparib and iniparib-met.

**Tables**

Table I. The IC$_{50}$s in the PARP-1/2 enzymatic assays and EC$_{50}$s in the PARP cellular assays were determined for the indicated compounds as described in Materials and Methods.
**Figures**

Figure 1. Compounds used in this study and their activity in PARP auto-modification assays.

(A) The structures of veliparib, cmpd-A, cmpd-B, cmpd-C, iniparib, and iniparib-met are shown. (B) *In vitro* PARP auto-modification assay was carried out for DMSO, iniparib, iniparib-met and comp-B as described in Materials and Methods. In the presence of NAD⁺, PARP-1 is poly-ADP ribosylated resulting in the appearance of a smear on the gel. Cmpd-B, but not iniparib or iniparib-met was able to prevent this effect.

Figure 2. Activity of veliparib, cmpd-A, iniparib, and iniparib-met in BRCA deficient and proficient cancer cells

(A, B) MDA-MB-436 (A) (2000 cells/well) and MDA-MB-231 (B) (500 cells/well) cells were plated into 96 well culture plates on day 1. On day 2, the indicated compounds were added and cells were allowed to grow for additional nine days. Cell proliferation was measured by cell titer glow assay as described in Materials and Methods. (♦ veliparib, ■ cmpd-A, ▲ cmpd-C, □ iniparib, ○ iniparib-met). (C, D, E, F) MDA-MB-436 (C, D) (4000 cells/well) and MDA-MB-231 (E, F) (1000 cells/well) cells were plated into 96 well plates and incubated with the indicated concentrations of iniparib or iniparib-met in the absence or presence of the indicated concentration of BSO for 5 days followed by cell titer-glow assay. (♦ 0 µM BSO, ■ 1.8 µM BSO, ▲ 3.75 µM BSO, □ 7.5 µM BSO). (G, H) Colony formation assays were carried out for DLD1-/- (G) or DLD1+/+ (H). Cells were plated into 6 well plates (200 cells/well) on day 1. The cells were treated with
the indicated concentration of veliparib or iniparib on day 2 for additional eleven days. The colonies were analyzed as described in Materials and Methods. (♦ veliparib, ■ iniparib).

Figure 3. Combination activity with TMZ

(A, B, C, D, E) MDA-MB-436 cells (4000 cells/well) were treated with indicated concentrations of TMZ and the indicated concentrations of veliparib (A), cmpd-A (B), cmpd-C (C), iniparib (D), and iniparib-met (E) for 5 days. Cell titer glow assays were carried out as described in materials and methods. (♦ 0 µM, ■ 0.005 µM, ▲ 0.05 µM, □ 0.5 µM, ○ 5 µM). (F, G, H, I) DLD1+/+ (F, H) or DLD1-/- cells (G, I) were plated into 96 well plates (1000 cells/well) and treated with indicated concentration of TMZ and the indicated concentration of veliparib (F, G) or iniparib (H, I) for 5 days. Cell titer glow assay was carried out as described in Materials and Methods. (♦ 0 µM, ■ 0.005 µM, ▲ 0.05 µM, □ 0.5 µM, ○ 5 µM).

Figure 4. Veliparib selectively attenuates DNA repair in DLD1-/- relative to DLD1+/+ cells. DLD1+/+ and DLD1-/- cells (0.3 million/well) were treated with DMSO, 0.1 mM TMZ, 5 µM veliparib, or 0.1mM TMZ and 5 uM veliparib for 30 min. Cells treated with DMSO or TMZ alone were pre-treated with DMSO for 30 min. Cell treated with veliparib alone or TMZ/veliparib combination were pre-treated with 5 µM veliparib for 30 min. The cells were then washed and incubated in complete medium containing either DMSO (for DMSO or TMZ alone) or 5 µM veliparib (for veliparib alone or TMZ/veliparib combination) for one hour or 6 hours. The γH2AX assay was carried out as described in Material and Methods. P values are indicated (Student’s T test).
Figure 5. Veliparib, but not iniparib, inhibits capan-1 xenograft tumor growth as single agent, and potentiates TMZ in B16F10 xenograft tumors.

(A) Single agent therapy was administered PO, bid x 14 days for veliparib and IP, bid x 14 days for iniparib on days 24-37. The experiment consists of 10 mice per treatment group; bars, SE. (B) TMZ was administered on a PO, q.d.x5 schedule on days 6-10 at 50 mg/Kg/d concurrently with veliparib on a PO, b.i.d.x5 schedule at 25 mg/Kg/d or with iniparib administered IP bid x 5 schedule at 25, 50 and 100 mg/Kg/d. The experiment consists of 10 mice per treatment group; bars, SE.

Figure 6. Iniparib-met reacts non-specifically with proteins containing cysteine residues 1 µM of the indicated proteins were incubated with 200 µM of the indicated compounds for two hours at RT in the presence of 50 mM HEPES and 100 mM KCl. Intact protein molecular weights were measured by mass spectrometry.

Figure 7. Iniparib and iniparib-met forms protein adducts non-specifically inside cells.

On day 0, 1 million cells per well were seeded into 6 well plates. On day 1, cells were treated with 30 µM iniparib with 0.1 mCi ³H-labeled iniparib or treated with 30 µM iniparib-met with 0.1 mCi ³H-labeled iniparib-met in the absence or presence of 0.5 mM BSO for 18 hours. Cells were harvested and directly lysed in 100 µl of lysis buffer. After centrifugation, 20 µl of the lysate was subjected to a 10% SDS-PAGE and transferred to a PVDF membrane. After PPO treatment, the membrane was exposed to film for 14 days. The same cell lysate was also subjected to western blot analysis for PARP-1 as described in Materials and Methods (bottom panel).
References


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Table 1
Fig. 1

DMSO

Iniparib

Iniparib-met

cmpd-B

NAD^+:

- + - + + +

100 kDa

150 kDa

PARP-1

PARylated PARP-1

Veliparib cmpd-A cmpd-B cmpd-C

Iniparib

Iniparib-met

Fig. 2
Fig. 3
Fig. 3
Fig. 4

A

B

DMSO
Veliparib
TMZ
TMZ + Veliparib
DMSO
Veliparib
TMZ
TMZ + Veliparib

γH2AX (% positive)

P = 0.021
P = 0.029
P = 0.078
P = 0.11

DLD1+/+
DLD1−/−

DLD1+/+
DLD1−/−

P = 0.01
P = 0.085
P = 0.39
P = 0.24

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**Fig. 5**

**A**

- **Vehicle**
- **Veliparib (200)**
- **Iniparib (100)**

Max % Wt Loss

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- 1500
- 1750

**B**

- **Vehicle**
- **TMZ**
- **Veliparib/TMZ (25/50)**
- **Iniparib/TMZ (100/50)**
- **Iniparib/TMZ (50/50)** N=9
- **Iniparib/TMZ (25/50)**

Mean Tumor Volume (mm³)

- 0
- 250
- 500
- 750
- 1000
- 1250
- 1500
- 1750

*p < 0.05 vs. vehicle

**Day**

- 0
- 20
- 30
- 40
- 50
- 60
- 70
- 80

**Day**

- 5
- 10
- 15

**Mean Tu**

- * p < 0.05 vs. vehicle

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Fig. 6
Fig. 7

**PARP-1**

- 25 kDa
- 37 kDa
- 50 kDa
- 75 kDa
- 100 kDa
- 150 kDa
- 200 kDa

**Iniparib**
- MDAMB231
- MDAMB436

**Iniparib + BSO**
- MDAMB231
- MDAMB436

**Iniparib - met**
- MDAMB231
- MDAMB436

**Iniparib - met + BSO**
- MDAMB231
- MDAMB436

**3H-labeled proteins**

**PARP-1**
Iniparib non-selectively modifies cysteine-containing proteins in tumor cells and is not a bona fide PARP inhibitor

Xuesong Liu, Yan Shi, David X Magg, et al.

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